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Bescheinigung | Certificate | Attestation

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

98201253.6

Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

DEN HAAG, DEN
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Page 2 de l'attestation

Anmeldung Nr
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Bezeichnung der Erfindung
Title of the invention
Titre de l'invention:

Method for the rapid determination of bacteria

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

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Bemerkungen
Remarks
Remarques

The title of the application as originally filed reads as follows:
Rapid bacterial determination

Eur4600

Title: Rapid bacterial determination.

The invention relates to the detection, identification or determination of bacteria in samples in general and in particular in clinical samples such as blood, urine, saliva, cerebrospinial fluid, faeces, pus and tissue that are taken from patients that are possibly infected with a, as yet unknown, possibly pathogenic bacterium, or during follow-up diagnostic testing to for example evaluate therapeutic measures that have been taken so far to treat the disease.

Traditional methods to determine or identify bacteria in general start with a Gram-stain, which is well known in the art. Such a stain can be performed on a sample immediately after sampling or, when not enough bacteria are present, after a short period of culturing of the sample. In general, four types of bacteria are found after Gram-staining; Gram-negative rods and cocci and Gram-positive rods and cocci. However, such a Gram-stain can only in very exceptional cases provide the clinician with the knowledge required to provide accurate therapy.

Examples of Gram-negative rods in clinical samples are *Enterobacter, Klebsiella, Salmonella, Escherichia, Proteus* and *Pseudomonas species*, of Gram-negative cocci are *Neisseria species*. Gram-positive rods that may be found in clinical samples are *Bacillus species*, of Gram-positive cocci are *Enterococcus, Streptoccus* and *Staphylococcus species*. Some of these, such as *Streptoccus* and *Staphylococcus* can easily be further determined or distinguished from each other by their morphological characteristics. *Streptococci* (and *Enterococci*) are

[illegible] clumps. Most other species, such as *Enterococcus* and

is affected also. This side-effect heavily decreases the patients defence against microbial invaders from the environment. Especially the lowering of the colonisation threshold of the gastro-intestinal tract may cause severe overgrowth by e.g. yeasts and fungi. The resulting secondary infection, or super-infection, in septicaemic patients who already suffer from a decreased immunity often leads to life-threatening situations.

Apart from the serious danger to the patient's health, wide-spectrum antibiotic therapy poses another threat. The repeated exposure of indigenous bacteria to antibiotics enhances the emergence of resistance against such an antibiotic. Especially when a resistance-gene is encoded on a plasmid, other (potential pathogenic) bacterial species may become resistant after the uptake of the plasmid. This latter scenario is considered to be a major problem in hospital epidemiology. It is therefor of paramount therapeutic and epidemiological importance to speed up the methodological procedures in the diagnosis of blood samples from for example septicaemic patients to be able to select specific antibiotic therapy designed for the specific pathogen found, thereby refraining from using broad-spectrum antibiotics.

Also, in those cases where resistance of a pathogen to antibiotics has already occurred, it is of utmost importance to be able to rapidly identify the pathogen and rapidly select the antibiotic against which the pathogen is not resistant.

In short, there is a need for fast and reliable diagnosis of bacteria, present in for example clinical samples that may replace or add to the currently used culturing techniques. Present techniques other than culturing, albeit in general specific when beforehand knowledge exists about the species involved, cannot be used with samples containing uncharacterised species or

First of all, hybridisation requires permeabilisation of the pathogen, and until now no generally applicable permeabilisation protocols have been developed that allow sufficient but restricted lysis of many or all of a broad range of unidentified bacteria. In general, mild permeabilisation leaves many bacteria (such as *Staphylococcus spec.*) inaccessible for subsequent hybridisation with probes, whereas rigorous permeabilisation often fully lyses most bacteria, thereby foregoing the possibility to detect them all together.

In addition, current protocols are in general time-consuming multi-step procedures; hybridisation often requires minimally 24 hours, thereby giving no relief to the needs of the clinician who is only helped with accurate and speedy diagnosis. Furthermore, they mostly require beforehand knowledge about the genus or even species involved in order to select appropriate probes; having such beforehand knowledge is clearly not the case in the event of a patient having an unidentified infection. Also, the present, already inappropriate hybridisation techniques do not allow to gather information on the response against antibiotics of the bacterium involved.

The invention provides a fast and reliable method for diagnosis, detection and/or determination of bacteria which may be present in a sample. Such a sample may be of various origin, it is for instance possible to apply a method as provided by the invention to a sample obtained from a (contaminated) bacterial culture, or drinking water or food suspected to be contaminated with a bacterium.

In a preferred embodiment the invention provides a method to detect or identify a bacterium suspected of being present in a clinical sample. Herein the term sample is meant a sample from a human, preferably a human more preferably human being. Such a sample may be sampled or tested

determining the rod or coccus character of said bacterium, thereby establishing the subsequent testing protocol.

When a Gran-negative bacterium is of the rod type, the invention provides a method further comprising hybridising said sample with at least one probe selected from a group of probes capable of hybridising with nucleic acid found in *Escherichia coli*, in *Klebsiella pneumoniae*, in *Klebsiella oxytoca*, in *Serratia marcescens*, in *Enterobacter aerogenes*, in *Enterobacter cloacae*, in *Proteus vulgaris*, in *Proteus mirabilis*, in *Salmonella typhi*, in *Pseudomonas aeruginosa*.

Furthermore, the invention provides a method wherein said character is of the Gram-negative coccus type, further comprising subjecting said sample to treatment with a lysis buffer comprising lysozyme. Also, when said Gram-staining indicates the presence of a Gram-positive bacterium in said sample, said method is further comprising determining the rod or coccus character of said bacterium, and when said Gram-positve character is of the rod type, further comprising subjecting said sample to treatment with a lysis buffer comprising lysozyme and/or Proteinase K.

In addition, when said character is of the Gram-positive coccus type, a method is provided further comprising determining a chain-like or clump-like character of said bacteria before a hybridisation protocol is selected. When before mentioned character is chain-like, a method provided by the invention is further comprising subjecting said sample to treatment with a lysis buffer comprising lysozyme, and further comprising hybridising said sample with at least one probe selected from a group of probes capable of hybridising with nucleic acid found in *Enterococcus faecalis*, in *Streptococcus pneumoniae*, in *Streptococcus mitis*, in *Streptococcus viridans*, in *Streptococcus sanguis*, in *Enterococcus faecium*.

majority of bacterial species. Preferably said majority comprises at least 90% of bacterial species, especially with those species found in general with possibly infected (septicaemic) patients. A method as provided by the invention is even more specific and/or sensitive when at least 95%, preferably at least 99% of said species is reactive with said positive control probe or no more than 5%, preferably no more than 1% is reactive with said negative control probe.

Such a positive or negative control probe as provided by the invention is given in the experimental part, in general said positive control probe comprises no more than five mismatches with a probe with the sequence GCTGCCTCCCGTAGGAGT and/or said negative control probe comprises no more than five mismatches with a probe with the sequence ACTCCTACGGGAGGCAGC.

Furthermore, the invention provides a method with additional value to the clinician in that in said method a probe is selected for its reactivity with one or a group of bacterial genera and/or (sub)species having congruent susceptibility to antibiotic treatment. Such a probe detecting or identifying a bacterium in a sample, preferably a clinical sample, is capable of hybridising with nucleic acid found in a group of bacterial genera and/or species or subspecies such as found with Staphylococcus and many other bacteria having congruent susceptibility to antibiotic treatment.

In a preferred embodiment of the invention, such a probe is having no more than five, preferably no more than two mismatches with a probe selected of a group composed of probes having a sequence GCCTGCCAGTTTCGAATG or GAGCAAAGGTATTAACTTTACTCCC (i.e. reactive with bacteria for which amoxycillin treatment is most likely effective)

or TTATCCCCCTCTGATGGG or GCCACTCCTCTTTTTCCGG (amoxycillin)

The invention is further explained in the experimental part of the description which is not limiting the invention.

Experimental part

An example of a set of probes specific for the detection of pathogenic bacteria and an example of a new protocol for high-speed in situ hybridisation are presented. The methodology described here is for example used for both a preliminary screening of samples from septicaemic patients or as a full substitute on the basis of which therapeutic decisions are made. The invention thus provides a rapid and reliable method for determining the species and/or the genus of a bacterium present in a blood sample collected from a septicaemic patient.

Components

A set of fluorescently labeled oligonucleotide probes designed to hybridise specifically with a group of pathogenic bacteria (i.e. *genus*-specific probe) or with one specific pathogen (i.e. *species*-specific probe) or with bacteria with congruent susceptiblity or resistance to antibiotics.

A protocol for fast *in situ* hybridization of bacteria present in samples of blood collected from septicaemic patients, using the said probes.

Oligonucleotide probes designed to hybridize specifically with a group of pathogenic bacteria.

In a particular embodiment of the invention a method provided by the invention is exemplified by making use of 16S rRNA target molecule because a large databank containing 16S rRNA-sequences exists and is freely

- Enzymes such as alkaline phosphatase or horseradishperoxidase either attached directly or via a C6-thiol linker and used in combination with chemiluminescent substrates like AMPPD (3-('spiroadmantane)-4-methoxy-4-(3'-phosphoryloxy)-phenyl-1,2-dioxethane) or fluorescence generating substrates.
- Digoxigenin (DIG) in combination with anti-DIG antibodies labeled with:
  - gold particles
  - fluorescent labels
  - Enzymes such as alkaline phosphatase or horseradish peroxidase, optionally in combination with chemiluminescent substrates like AMPPD (3-('spiroadmantane)-4-methoxy-4-(3'-phospho- ryloxy)-phenyl-1,2-dioxethane) or fluorescence generating substrates.
- Biotin in combination with streptavidin or avidin and labeled just like the anti-DIG antibodies
- Dinitrophenyl as hapten in combination with appropriate antibodies and labeled just like the anti-DIG antibodies
- Any other indirect fluorescent or enzymatic label

Fluorescent labels allow direct microscopic analysis preferably combined with image analysis. For the detection of fluorescent oligonucleotide probes hybridised to ribosomal RNA of the target bacterium, photography can be applied. However quantitation by this method is hampered by the absence of objective criteria by means of which discrimination between hybridized and non-hybridized cells can be performed. Therefore for objective evaluation of probe-specificity, an image analysis system is employed which allows fluorimetrical reading of individual

present in samples of blood

hybridisation time of minimally 24 hours, rendering them useless for rapid diagnostic applications.

The invention also provides kits for carrying out the rapid detection of bacteria in blood samples according to the invention. Such a kit will usually comprise at least a probe or probes and optionally other reagents such as components for hybridisation-fluid, washing-fluid and permeabilisation-fluid.

Such a kit may be applied in a routine bacteriology laboratory or in a bedside environment, both as a fast screening method or as a full substitute for classical identification methods.

Examples of probe design and development

The following probes for example were found to hybridise the most predominant species of pathogens which are found in blood from septicaemic patients. In addition each probe hybridises with a species or a cluster of bacteria which share congruent (but often not identical) antibiotic sensitivity patterns

ID	Sequence (5'-3')[1]	Region[2]	Specificity[3]
A	GCTGCCTCCCGTAGGAGT	V2	Bacterial Kindom
B	ACTCCTACGGGAGGCAGC	n.d.	no matches
C	GCCTGCCAGTTTCGAATG	V2	Salmonella spp, Klebsiella spp, Enterobacter spp.
D	GTAGCCCTACTCGTAAGG	V7	K. oxytoca, S. marcescens, Enterobacter spp, Proteus spp
E	GAGCAAAGGTATTAACTTTACTCCC	V3	E. coli
F	TTATCCCCCTCTGATGGG	V2	E. faecalis
G	GCTAATGCAGCGCGGATCC	V2	S. aureus, S. haemolyticus
H	CCGAAGGGGAAGGCTCTA	V6	S. aureus, S. saprophyticus
I	AGAGAAGCAAGCTTCTCGTCCG	V1	Streptococcus spp.
J	GTTAGCCGTCCCTTTCTGG	V3	P. aeruginosa
K	AGAGAAGCAAGCTTCTCGTCCGTT	V2	S. aureus
L	GCCACTCCTCTTTTTCCGG	??	Enterococcus faecium

9 Permeabilise **Staphylococci** 20 min at 25 C with Lysostaphin (100 units/ml)

10 Rinse the slide with (demineralised) water for 5 minutes

11 Dry the slide for 5 minutes on a hotplate.

12 Pipet hybridisation buffer(+SDS)-probe mix ([probe]=10ng/µl). Cover with a coverslip.

13 Hybridize 2 hours (for example at 48°C).

14 Rinse 5 min using hybridisation buffer(-SDS).

15 Mount the slide with a coverslip.

16 Evaluate the slide.

phosphate buffered saline

8 g/l NaCl
- 0.2 g/l KCl
- 1.44 g/l Na_2HPO_4
- 0.24 g/l KH_2PO_4
- adjust to pH 7.4.

Hybridisation buffer (+SDS)

- 900 ml Milli-Q water
- 52.6 g NaCl
- 2.52 g Tris (hydroxymethyl)-aminomethane
- adjust to pH 7.5
- add 90 ml water.
- sterilize 15 minutes
- 10 ml SDS (10%) stock

hybridisation buffer (-SDS)

- 900 ml Milli-Q water
- 52,6 g NaCl
- 2,52 g Tris

sterilize 15 minutes

predominant bacteria in sepsis were hybridised using both the protocol and the probes mentioned here above. The result of this validation is listed in table 2. As can be read from this table all probes yield a satisfying hybridisation profile. Using the group-probes C and D it is possible to distinguish between: four groups of gram-negative rods:

C-positive and D-positive: Klebsiella oxytoca, Enterobacter cloacae and Enterobacter aerogenes

C-positive and D-negative: Klebsiella pneumoniae and Salmonella typhi

C-negative and D-positive: Serratia marcescens and Proteus vulgaris

C-negative and D-negative: Proteus mirabilis.

For Escherichia coli and Pseudomonas aeruginosa two *species*-specific probes (E and J) have been designed and validated. These probes are optionally included because both Escherichia and Pseudomonas are notorious pathogens which demand specific antimicrobial therapy. Probe F is a *species*-specific probe for Enterococcus faecalis, a notorious pathogen. Probe I is a *genus*-specific probe which can be used in conjunction with probe F because Streptococci and Enterococci share the same morphology, while they require different antimicrobial treatment.

Using both probes G and H, 4 separate species of Staphylococci can be distinguished:

G-positive and H-positive: Staphylococcus aureus

G-positive and H-negative: Staphylococcus haemolyticus

G-negative and H-positive: Staphylococcus saprophyticus

G-negative and H-negative: Staphylococcus epidermidis

Probe K is a *species*-specific-probe for Staphylococcus aureus and can be used to support the results obtained by probes G and H.

CLAIMS

1. A method for determining a bacterium suspected of being present in a sample comprising
a) testing said sample by Gram-staining and
b) testing said sample with a probe according to an *in situ* hybridisation protocol selected on the basis of the outcome of said Gram-staining.

2. A method according to claim 1 wherein said sample is a clinical sample.

3. A method according to claim 2 wherein said sample is mammalian blood, preferably being derived from a human.

4. A method according to claim 1, 2 or 3 wherein said Gram-staining indicates the presence of a Gram-negative bacterium in said sample, further comprising determining the rod or coccus character of said bacterium.

5. A method according to claim 4 wherein said character is of the rod type, further comprising hybridising said sample with at least one probe selected from a group of probes capable of hybridising with nucleic acid found in *Escherichia coli*, in *Klebsiella pneumoniae*, in *Klebsiella oxytoca*, in *Serratia marcescens*, in *Enterobacter aerogenes*, in *Enterobacter cloacae*, in *Proteus vulgaris*, in *Proteus mirabilis*, in *Salmonella typhi*, in *Pseudomonas aeruginosa*.

6. A method according to claim 5 wherein said nucleic acid is ribosomal RNA.

7. A method according to claim 6 wherein said probe is having no more than five, preferably no more than two mismatches with a probe selected of a group composed of probes having a sequence GCCTGCCAGTTTCGAATG or

8. A method according to claim 4 wherein said character is of the coccus type, further comprising subjecting said

from a group of probes capable of hybridising with nucleic acid found in *Staphylococcus aureus*, in *Staphylococcus haemolyticus*, in *Staphylococcus saprophyticus*.

18. A method according to claim 17 wherein said nucleic acid is ribosomal RNA.

19. A method according to claim 18 wherein said probe is having no more than five, preferably no more than two mismatches with a probe selected of a group composed of probes having a sequence GCTAATGCAGCGCGGATCC or CCGAAGGGGAAGGCTCTA or AGAGAAGCAAGCTTCTCGTCCGTT.

20. A method according to any of claims 4 to 19 further comprising hybridising said sample with at least one positive control probe and/or with at least one negative control probe.

21. A method according to claim 20 wherein said positive control probe comprises no more than five mismatches with a probe with the sequence GCTGCCTCCCGTAGGAGT and/or wherein said negative control probe comprises no more than five mismatches with a probe with the sequence ACTCCTACGGGAGGCAGC.

22. A method according to anyone of claims 1 to 21 further comprising a one-step procedure to bind bacteria present in said sample to a microscopic slide and simultaneously fix intracellular structures.

23. A method according to anyone of claims 1 to 22 wherein said probe is selected for its reactivity with one or a group of bacterial genera and/or species having congruent susceptibility to antibiotic treatment.

24. A probe detecting or identifying a bacterium in a sample, preferably a clinical sample, said probe capable of hybridising with nucleic acid found in a group of bacterial genera and/or (sub)species having congruent susceptibility to antibiotic treatment.

25. A probe according to claim 24 wherein said probe is having no more than five, preferably no more than two mismatches with a probe selected of a group composed of

ABSTRACT

The invention relates to the detection, identification and diagnosis of bacteria in samples in general and in particular in clinical samples such as blood, urine, saliva, cerebrospinal fluid that are taken from patients that are possibly infected with a, as yet unknown, possibly pathogenic bacterium, or during follow-up diagnostic testing to for example evaluate therapeutic measures that have been taken so far to treat the disease.

The invention provides a method for detecting or identifying a bacterium suspected of being present in a sample comprising testing said sample by Gram-staining and testing said sample with a probe according to an *in situ* hybridisation protocol selected on the basis of the outcome of said Gram-staining. The invention also provides probes for use in said method.